AMENDMENTS TO THE SPECIFICATION

Please amend the title as follows:

"~~Caspase-9 BIR3 Domain of XIAP Complexes~~ Inhibitors of Initiator Caspases and Methods of Use".

Please amend the abstract as follows:

The present invention provides polypeptides and specific binding agents that ~~modify the activity of an~~ inhibit initiator ~~caspase~~ caspases involved in apoptosis, such as, for example, human caspase-9, and methods of making and using such inhibitors. ~~The polypeptides include the third baculoviral IAP repeat (BIR3) of an IAP and form a heterodimer complex with caspase-9.~~ Nucleic acid molecules including expression vectors encoding the polypeptides and variants of such inhibitors are also provided. ~~thereof as well as variants of caspase-9 are provided. Such polypeptide and nucleic acid molecules may be used for modifying apoptosis.~~

Please amend paragraph [0001] as follows:

[0001] This application claims the benefit of and priority to U.S. Provisional Application Serial Number 60/443,590 filed January ~~30~~ 31, 2003, the contents of which are incorporated herein by reference in ~~their~~ its entirety.

Please amend paragraph [0029] as follows:

[0029] Through crystallization and structure determination it was determined that the BIR3 domain of XIAP readily forms a tight complex with caspase-9, (SEQ ID NO:6), and inhibits its catalytic activity with a potency similar to that of the intact full-length XIAP (SEQ ID NO:13). X-ray ~~crystallograpy~~ crystallography is one method that could be used to determine the structure and binding sites of other specific binding agents with initiator caspases like caspase-9. The structure of caspase-9 with various polypeptides, peptidomimetics, their variants, and point mutants may be determined using the methods disclosed herein. In the present invention, the mechanism of XIAP-mediated inhibition of caspase-9, was determined through the crystal structure of a caspase-9/XIAP-BIR3 complex (SEQ ID NO:6). It was possible to generate crystals of the catalytic domain of caspase-9 (residues ~~139-416~~ 140-416) in an inhibitory complex with the XIAP-BIR3 domain (residues ~~252-350~~ 253-350). The crystals in this

inhibitory complex are in the spacegroup P6$_{2}$22 and diffract X-rays beyond 2.4 Å resolution (Table 1). The caspase-9 moiety in the asymmetric unit was located by Molecular Replacement using the atomic coordinates of the active half of the ~~caspse~~ caspase-9 dimer as the initial search model (PDB code 1JXQ). The electron density for the bound BIR3 domain became immediately apparent after preliminary refinement. The final atomic model of the inhibitory complex has been refined to a crystallographic R factor of 23.0% (R_{free}23.5%) at 2.4 Å resolution (Table 1).

Please amend paragraph [0077] as follows:

[0077] This example describes the preparation of proteins, polypeptide, and the preparation of caspase-9 variants of the present invention. All constructs were generated using a standard PCR-based cloning strategy, and the identities of individual clones were verified through double stranded plasmid sequencing. To minimize self-cleavage in bacteria, the catalytic subunit of caspase-9 (residues ~~139-416~~ 140-416, in vector pET-21b) was co-expressed with the BIR3 domain of XIAP (residues ~~252-350~~ 253-350, in vector pBB75) in *Escherichia coli* strain BL21(DE3). A serendipitous bonus from this co-expression is a large quantity of unprocessed procaspase-9 zymogen. The soluble fraction of the caspase-9/BIR3 complex and the procaspase-9 zymogen in the *E. coli* lysate were purified using a Ni-NTA (Qiagen) column, and further fractionated by anion-exchange (Source-15Q, Pharmacia) and gel-filtration chromatograph (Superdex-200, Pharmacia). ~~Recombiant~~ Recombinant active caspases-7 and missense mutant of caspase-9 and XIAP-BIR3 were over-expressed and purified as described (Chai et al., 2001a; Chai et al., 2001b). For the three caspase-9 deletion variants (FIG. 4A), the large and the small subunits were co-expressed and purified as described (Chai et al., 2001b).

Please amend paragraph [0039] as follows:

[0039] Mutational analysis was used to corroborate this structural observation, a caspase-9 assay was ~~devise~~ devised using its physiological substrate, procaspase-3 zymogen, and the ability of various XIAP-BIR3 point mutants to inhibit caspase-9 was investigated. Similar tests could be used to determine the activity of other specific binding agents such as polypeptides, peptidomimetics, their variants, and point mutants. A mutation on the catalytic residue, Cys163 to Ala, was introduced in the substrate procaspase-3 to prevent its self-activation or cleavage. As anticipated, the wild type (WT) caspase-9 cleaved the procaspase-3 precursor

into p17 and p12 fragments (FIG. 3E, lane 1) and incubation with the WT BIR3 protein (residues ~~252-350~~ 253-350) resulted in the efficient inhibition of the activity (lane 2). In contrast to the WT protein, mutation of any of the four caspase-9 activity inhibiting amino acid residues of BIR3 (P325G, G326E, H343A, and L344A) led to loss of this inhibition as judged by the cleavage of procaspase-3 precursor (FIG. 3E, lanes 4, 5, 8, and 9). The result that H343A can no longer inhibit caspase-9 confirms an earlier report. These residues make important contributions to the recognition and sequestration of the caspase-9 monomer (Figures 3B-3D); mutation of any of these residues presumably destabilizes the interface, allowing the caspase-9 restoration of its catalytic activity. It is of particular note that none of these mutations affects the conserved surface groove on BIR3; thus caspase-9 is still able to bind to the mutated BIR3 domain but is no longer subject to its inhibition.

Please amend paragraph [0083] as follows:

[0083] Analytical ultracentrifugation. Protein samples were prepared in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mm DTT. All sedimentation equilibrium experiments were carried out at 4° C using a Beckman Optima XL-A analytical ultracentrifuge equipped with an An60 Ti rotor and using six-channel, 12 mm path length, charcoal-filled Epon centerpieces and quartz windows. Data were collected at four rotor speeds (10,000, 15,000, 20,000, and 25,000 rpm) and represent the average of twenty scans using a scan step-size of 0.001 cm. Partial specific volumes and solution density were ~~calculate~~ calculated using the Sednterp program. Data were analyzed using the WinNONOLIN program from the Analytical Ultracentrifugation Facility at the University of Connecticut (Storrs, CT). The results show that caspase-9 exists mostly as a monomer in solution and a single species of caspase-9 has been observed in solution by gel filtration as well as by analytical ultra-centrifugation.

Table 1. Data collection and statistics from the crystallographic analysis

Beamline	CHESS-A1
Spacegroup	$P6_522$
Resolution (Å)	99.0 – 2.3 Å
Total observations	415,375
Unique observations	23,136
Data coverage (outer shell)	99.7% (100%)
R_{sym} (outer shell)	0.071 (0.525)
Refinement:	
Resolution range (Å)	20.0 – 2.4 Å
Number of reflections (all)	22104
Data coverage	100%
$R_{working}/R_{free}$	0.230 / 0.235
Number of atoms	2806
Number of waters	215
R.m.s.d. bond length (Å)	0.012
R.m.s.d. bond angles (degree)	2.09
Ramachandran Plot:	
Most favored (%)	84.6
Additionally allowed (%)	14.3
Generously allowed (%)	1.1
Disallowed (%)	0.0

$R_{sym} = \Sigma_h \Sigma_i | I_{h,i} - I_h | / \Sigma_h \Sigma_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry related reflections of h. $R = \Sigma | F_{obs} - F_{calc} | / \Sigma F_{obs}$, where $F_{obs} = F_p$, and F_{calc} is the calculated protein structure factor from the atomic model (R_{free} was calculated with 5% of the reflections). R.m.s.d. in bond lengths and angles are the deviations from ideal values, and the r.m.s.d. deviation in B factors is calculated between bonded atoms.

Table 2. A summary of the analytical ultracentrifugation measurements.

Sample	Concentration	Molecular Weight (Dalton) Observed	Calculated
Caspase-9 (active)			
	20 μM	28,500±700	31,297
	10 μM	31,120±1,540	31,297
Caspase-9/XIAP-BIR3			
	20 μM	39,380±1,220	42,973
	10 μM	41,060±1,530	42,973
	5 μM	42,200±2,440	42,973
Caspase-7			
	20 μM	54,530±1,070	29.865
	10 μM	49,720±1,070	29,865
Procaspase-9 zymogen			
	20 μM	29.920±1,400	31,457
	10 μM	27,840±2,150	31,457

Molecular weight represents global analysis of data collected at four rotor speeds 10K, 15K, 20K, 25K rpm. All data were collected at 4°C. The ~~Caspase-9/XIAP-BIR3~~ caspase-9/XIAP-BIR3 sample contains the wild-type caspase-9 residues 139-315 and 316-416 and XIAP residues ~~252-350~~ 253-350. The active caspase-9 contains residues 139-315 and 316-416 except that residues Glu304-Asp305-Glu306 have been replaced by three Ala residues to reduce limited

proteolysis by the intrinsic enzymatic activity of caspase-9. The procaspase-9 zymogen contains residues 139-416. The active caspase-7 contains residues 51-198 and 200-303.